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Impact of Human Immunodeficiency Virus Infection in Pregnant Women on Variant-Specific Immunity to Malaria[▽]

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Human immunodeficiency virus (HIV) increases susceptibility to *Plasmodium falciparum* infection, and this has most clearly been demonstrated in pregnant women. Variant surface antigens on the surfaces of erythrocytes infected with *P. falciparum* are major targets of protective immunity. We studied the impact of HIV infection on pregnant women's humoral immunity to variant surface antigens expressed by placental and pediatric isolates of *P. falciparum*. By flow cytometry, sera from HIV-infected women more frequently lacked antibodies to these antigens than sera from HIV-uninfected women. This difference was similar in magnitude for pediatric isolates (unadjusted odds ratio [OR] = 6.36; 95% confidence interval [CI] = 1.14, 35.32; $P < 0.05$) and placental isolates (unadjusted OR = 6.47; 95% CI = 0.75, 55.64; $P < 0.10$). We divided women into high and low responders on the basis of their antibody levels. After adjustment for CD4 count, maternal age, and gravidity, we found that HIV-infected women more frequently had low responses to both pediatric isolates (OR = 5.34; 95% CI = 1.23, 23.16; $P = 0.025$) and placental isolates (OR = 4.14; 95% CI = 1.71, 10.02; $P = 0.002$). The relative quantity of antibodies to both pediatric isolates ($P = 0.035$) and placental isolates ($P = 0.005$) was lower in HIV-infected women than in HIV-uninfected women. HIV infection has a broad impact on variant-specific immunity, which may explain the susceptibility of infected individuals to clinical malaria episodes.

Plasmodium falciparum infection induces the expression of variant surface antigens (VSAs) on the red blood cell (RBC) surface. These VSAs form a highly diverse group of antigens and appear to be the primary targets of host immunity. Adults in areas where malaria is endemic are usually semi-immune to *P. falciparum*, with this immunity being associated with the acquisition early in childhood of antibodies directed against VSAs. Pregnant women (especially primigravidae) have more frequent and higher-density episodes of malaria parasitemia than their nonpregnant counterparts (3). This arises in part because infected RBCs (IRBCs) can sequester in the placenta by adhering to receptors such as chondroitin sulfate A (8), and these IRBCs express unique, pregnancy-specific VSAs to which primigravidae lack immunity.

Antibodies that target VSAs are associated with the protection of children from clinical episodes of malaria (6). In pregnant women, antibodies that inhibit *P. falciparum* adhesion to chondroitin sulfate A have been associated with increased birth weight and gestational age at delivery (7), and antibodies to total VSAs were associated with a reduced prevalence of maternal anemia and low birth weight (14). In a previous study, we found that human immunodeficiency virus (HIV) infection impairs pregnancy-associated variant-specific immunity, and the impairment was the greatest in women with low CD4 counts and high viral loads (11). This impairment of humoral immunity to pregnancy-specific VSAs may contribute to the increased susceptibility of HIV-infected (HIV^+) pregnant women to malaria and could explain their failure to demonstrate gravidity-dependent acquisition of immunity (reviewed in reference 15).

Studies indicate that *P. falciparum* infection increases the HIV viral load in pregnant women and nonpregnant adults (10, 12) and that HIV infection increases the incidence of *P. falciparum* infections and clinical malaria (13, 17). All of these data taken together suggest that malaria and HIV coinfection in pregnant women is a major public health concern.

Given the importance of immunity to VSAs in protection from clinical malaria (6) and our previous observation that HIV impairs immunity to pregnancy-associated VSAs, we further examined the impact of HIV infection on immunity. Using a single panel of sera from HIV^+ and HIV-negative (HIV^-) women, we compared the prevalence and relative quantity of antibody to VSAs expressed by isolates from the placenta and isolates from children with symptomatic malaria to discover whether the impairment of immunity to VSAs was restricted to pregnancy-associated malaria or whether a more general defect in immunity to VSAs existed.

MATERIALS AND METHODS

Sample selection and processing. *P. falciparum* isolates were obtained from children admitted between October 2004 and April 2006 to the pediatric ward of the Queen Elizabeth Central Hospital, Blantyre, Malawi, with severe and complicated malaria and parasitemia of 3% or more on thin blood film examination. Before treatment was begun, a sample of peripheral blood (300 to 500 μl) was

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TABLE 1. Maternal antibody recognition of placental and pediatric isolates[a]

Isolate type	First comparison					Second comparison				
	Antibody response	No. of results for group[b]		OR (95% CI)	*P* value	Antibody response	No. of results for group[b]		OR (95% CI)	*P* value
		HIV^+	HIV^-				HIV^+	HIV^-		
Pediatric	Absent	44	11	5.89 (0.84, 41.27)	0.074	Low	71	36	5.34 (1.23, 23.16)	0.025
	Present	56	89			High	29	64		
Placental	Absent	6	1	5.78 (0.47, 70.63)	0.169	Low	54	31	4.14 (1.71, 10.02)	0.002
	Present	64	69			High	16	39		

[a] Antibody was defined as present (MFI values greater than the mean + 2 SDs of the negative controls) or absent (MFI values less than or equal to the mean + 2 SDs of the negative controls) (first comparison) and high (MFI > 50 units) or low (MFI ≤ mean of 50 units) (second comparison). The OR and the 95% CI of the failure of HIV^+ sera to recognize IRBCs from both pediatric and placental isolates were computed by using generalized estimating equations to address the clustering by study subject and adjusted for CD4 count (<200/μl and ≥200/μl), maternal age (≤24.5 and >24.5 years), and gravidity (one or two pregnancies and three or more pregnancies).

[b] Twenty serum samples, 10 from HIV^+ women and 10 from HIV^- women, were tested against 10 pediatric isolates or 7 placental isolates.

collected in lithium heparin tubes (D-51588; Sarstedt AG & Co., Nümbrecht, Germany). The RBC pellet was washed three times in RPMI-HEPES medium and cultured in human blood group O-positive RBCs in RPMI-HEPES medium supplemented with 10% pooled human serum, 5.6% of 3.6 g/100 ml $NaHCO_3$, and 0.1% of 10 mg/ml gentamicin, as described elsewhere (11). The parasites were cultured for at least 24 h to a parasitemia of 3 to 10% pigmented trophozoites, as determined with Giemsa-stained thin blood films.

Placental isolates were extracted from freshly delivered placentas. Pieces of placental tissue (2 cm^3) were collected immediately upon delivery from consenting women giving birth in the labor ward of the Queen Elizabeth Central Hospital. These were placed in phosphate-buffered saline (PBS; pH 7.4) in 50-ml Falcon tubes. Placental parasites were dislodged from the tissue by incubation at room temperature for 2 h on a roller and three washes (1,500 rpm for 5 min each time) in RPMI-HEPES, as described previously (2). Because the initial levels of parasitemia varied quite widely, placental trophozoites were enriched to 90 to 98% trophozoites by using discontinuous 80%, 60%, and 40% Percoll gradients (GE Healthcare, Uppsala, Sweden) (www.malaria.mr4.org). The parasitemia was then reduced to approximately 10% with fresh group O-positive RBCs for flow cytometry so that the parasitemias for the pediatric isolates and the placental isolates were similar. Seven placental isolates and 10 peripheral blood isolates were used.

We randomly selected 20 maternal serum samples (10 HIV^+ and 10 HIV^-, all of which were of blood group O) from an existing study sample set (11, 12). Women had undergone voluntary counseling before and after HIV testing and had been informed of their HIV status.

Measurement of antibodies to VSAs by flow cytometry. *P. falciparum* IRBCs from placental or pediatric isolates at 3 to 10% parasitemia of trophozoites were washed three times in PBS containing 1% fetal calf serum (PBS–1% FCS) (11). Cells at 0.1% hematocrit (100 μl) were incubated with test sera at a 1:20 dilution in PBS–1% FCS for 30 min in 5-ml polystyrene round-bottom tubes (Falcon 2008; Becton Dickinson, Franklin Lake, NJ). This was followed by a 30-min incubation with rabbit anti-human immunoglobulin G (IgG; 50 μl; Dako A/S, Denmark) at a 1:100 dilution in PBS–1% FCS and a final incubation with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (heavy and light chains; Invitrogen, Eugene, OR) at a 1:500 dilution and 10 mg/ml ethidium bromide at a 1:100 dilution (50 μl) in the dark for 30 min. All incubations were performed at room temperature. After each incubation, the cells were washed three times in PBS–1% FCS. They were finally resuspended in 200 μl PBS and analyzed with a Becton Dickinson FACSCalibur flow cytometer with Cell Quest software. All samples were run together with a positive control made up of a pool of hyperimmune sera from pregnant women resident in Blantyre, Malawi, and two negative controls made up of pooled sera from adults in Melbourne, Australia, who had never been exposed to the malaria parasite. The geometric mean fluorescence intensity (MFI) was calculated as a measure of IgG binding to IRBCs (11). IRBCs were gated, and 1,000 positive cells were collected on the basis of ethidium bromide fluorescence. Samples were determined to have antibodies if the MFI was greater than the mean of the negative controls plus 2 standard deviations (SDs). Serum samples giving readings greater than the reading for the positive control were given a value of 100 units, and those giving a reading below the mean value of the negative controls were given a value of 0. Sample readings between the two controls were assigned relative values by using the formula (11) [(serum sample reading − negative control reading)/(positive control reading − negative control reading)] × 100.

To further assess the relationship between HIV infection and antibody levels, the latter were dichotomized into high and low responders by using a relative MFI of 50 units.

Data management. All data collected were entered into Excel software (Microsoft Co.) and were analyzed with the Statistical Package for Social Scientists (SPSS Inc., Chicago, IL), the Stata program (version 8.0; Stata Corporation, College Station, TX), and the SAS program (version 9.1; SAS Institute, Inc., Cary, NC). Generalized estimating equations with an exchangeable correlation matrix were used to generate odds ratios (ORs). These types of models allowed us to take into account the clustering that resulted from the consideration of multiple outcomes for each woman. We ran both unadjusted models and models that adjusted for CD4 count, maternal age, and gravidity. Fisher's exact tests were used to assess bivariate relationships. The levels of antibodies to VSAs were compared by the Mann-Whitney U test.

One of the HIV^+ women had missing CD4 count data; because we had only 10 HIV^+ subjects and wished to conduct a complete case analysis, we chose to impute the median CD4 value (178/μl) for this woman. None of the HIV^- women had CD4 count data; for the sake of including them in the adjusted models, we imputed a CD4 count of 500/μl for each woman. In all analyses, age was dichotomized at the median value (≤24.5 and >24.5 years); the CD4 count (<200/μl and ≥200 μl (4) and gravidity (one or two pregnancies and three or more pregnancies) were also dichotomized.

Ethical approval. This study was approved by the College of Medicine Research Committee, University of Malawi, Blantyre, Malawi; the Royal Melbourne Hospital Clinical Research Ethics, Melbourne, Victoria, Australia; and the University of North Carolina Institutional Review Board.

RESULTS

We used flow cytometry to measure antibodies to the VSAs of *P. falciparum* isolates from 10 children and seven placentas. HIV infection was associated with qualitatively and quantitatively lower levels of variant-specific immunity to both placental and pediatric isolates. The differences between the relative quantity of the antibody response in infected and uninfected women were statistically significant for pediatric isolates but not for placental isolates (Table 1; Fig. 1). Several serum samples lacked antibodies to pediatric isolates but had antibodies to placental isolates (Table 2).

We analyzed HIV infection status and the maternal antibody response (absent or present) to IRBCs separately for pediatric and placental isolates. The unadjusted odds of failure among the HIV^+ maternal sera to recognize pediatric isolates were six times the odds of failure among the HIV^- maternal sera (OR = 6.36; 95% confidence interval [CI] = 1.14, 35.32; *P* =

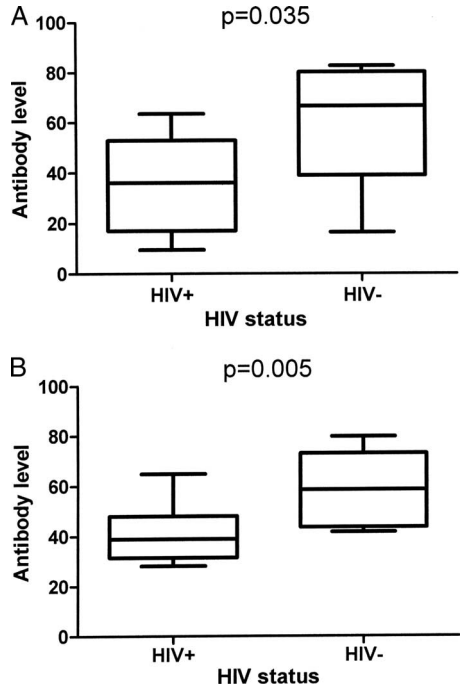

FIG. 1. Box and whisker plots of relative levels of antibodies to VSAs expressed by parasites infecting children (A) and parasites infecting the placentas of pregnant women (B) for HIV^+ and HIV^- pregnant women. The data summarize the results for 20 serum samples tested against 10 pediatric isolates (A) or against seven placental isolates (B). The boxes show the 25th to the 75th percentiles, the lines in the boxes show the median values, and the whiskers indicate the 95% range. *P* values were determined by the Mann-Whitney U test.

0.035). In the fully adjusted model, the relationship lost statistical significance at the 0.05 level, although none of the covariates were significant predictors (OR = 5.89; 95% CI = 0.84, 41.27; $P = 0.074$). In the case of placental isolates, the magnitudes of the associations in both the unadjusted model and the fully adjusted models were similar to those for the pediatric isolates, although neither reached statistical significance (*P* values of 0.089 and 0.169, respectively) (Table 1).

We conducted an additional analysis to assess the relationship between HIV infection status and antibody levels; we used a relative MFI value of 50 units (the midpoint of our measurement scale) to assign maternal antibody responses into high responders (MFI $>$ 50 units) and low responders (MFI $\leq$ 50 units). Fifty units may more closely reflect a protective level of antibody than the cutoff of the mean of our negative control plus 2 SDs. The unadjusted odds for having a low antibody response to pediatric isolates for HIV^+ maternal sera were four times the odds of having a low antibody response for HIV^- maternal sera (OR = 4.15; 95% CI = 1.40, 12.31; $P = 0.010$). That relationship remained significant in the fully adjusted model ($P = 0.025$). When the response to placental isolates was dichotomized into low versus high, the unadjusted odds for a low antibody response for HIV^+ sera were four times the odds for a low antibody response for HIV^- maternal sera (OR = 4.25; 95% CI = 2.30, 7.85; $P < 0.001$). That relationship also remained significant in the fully adjusted model ($P = 0.002$) (Table 1).

HIV^+ women had a median CD4 count of 178/μl. Among the HIV^+ women, we found no statistically significant association between the recognition of pediatric isolates and the CD4 count, dichotomized as high or low (Fisher's exact $P = 0.840$), nor did we find an association between the recognition of placental isolates and a high or a low CD4 count (Fisher's

TABLE 2. Maternal serum recognition of placental and pediatric isolates

Serum sample	Maternal age (yr)	Gravidity	HIV status[a]	CD4 count	Mal[b]	Antibody response to pediatric isolates[c]										Antibody response to placental isolates[c]						
						C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	P1	P2	P3	P4	P5	P6	P7
A	16	1	1	226	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	35	6	1	156	0	+	+	−	−	+	+	+	+	+	−	+	+	+	+	+	+	+
C	25	3	1	91	0	−	−	−	+	−	−	−	+	−	−	+	+	+	−	+	−	+
D	31	4	1	237	0	−	−	−	−	−	−	+	−	−	−	+	+	+	+	+	+	+
E	24	3	1	133	0	−	−	−	−	−	−	−	+	−	−	+	+	+	−	+	−	+
F	34	4	1	178	0	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+
G	26	1	1	—[d]	0	−	+	−	+	−	−	+	−	+	+	+	+	+	+	+	+	+
H	29	6	1	370	360	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+
I	19	1	1	170	7,140	+	+	+	+	−	−	−	−	+	−	+	+	+	+	+	−	−
J	20	1	1	265	6,540	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K	26	4	2	—	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
L	20	1	2	—	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M	40	7	2	—	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N	20	2	2	—	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O	19	1	2	—	27,960	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P	25	3	2	—	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Q	19	1	2	—	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
R	23	1	2	—	0	−	−	−	−	−	+	−	+	−	−	+	+	+	+	+	+	+
S	18	1	2	—	0	+	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+
T	32	8	2	—	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] 1, HIV^+; 2, HIV^-.
[b] Mal, number of malaria parasites detected on peripheral blood film.
[c] +, positive for recognition; −, negative for recognition.
[d] —, CD4 count not measured.

TABLE 3. Maternal antibody recognition of pediatric isolates based on gravidity and HIV status

Isolate type	Gravidity	HIV status	% in which antibody was present	*P* value[a]
Pediatric	Primigravidae/secundigravidae	Positive	75	0.460
	Primigravidae/secundigravidae	Negative	82	
	Multigravidae	Positive	43	<0.001
	Multigravidae	Negative	100	
Placental	Primigravidae/secundigravidae	Positive	93	0.560
	Primigravidae/secundigravidae	Negative	98	
	Multigravidae	Positive	90	0.144
	Multigravidae	Negative	100	

[a] HIV^+ versus HIV^-.

exact $P = 1.000$). Among all study participants, maternal age was not statistically significantly associated with the antibody response to pediatric isolates (Fisher's exact $P = 0.527$) or placental isolates (Fisher's exact $P = 0.441$).

When we evaluated the recognition of pediatric and placental isolates by HIV infection status and gravidity, differences between primigravid/secundigravid women and multigravid women emerged (Table 3). HIV^- sera from primigravid/secundigravid women recognized 82% of the pediatric isolates, which was similar to the value of 75% for the HIV^+ sera (Fisher's exact $P = 0.460$). However, among the multigravidae there was a striking difference. One hundred percent of the HIV^- sera recognized the pediatric isolates, but only 43% of the HIV^+ sera recognized the same isolates (Fisher's exact $P < 0.001$). There were no significant differences in response to placental isolates when the responses were stratified by gravidity. Sera from HIV^- primigravid or secundigravid women recognized 98% of the isolates, as did sera from 93% of the HIV^+ women (Fisher's exact $P = 0.560$). Among the multigravid women, 100% of the HIV^- women and 90% of the HIV^+ women recognized the same isolates ($P = 0.144$). Taken together, these findings suggest that HIV^+ pregnant women have lower levels of immunity to VSAs and that this difference is greater for immunity to variants expressed in pediatric malaria than for immunity to placental isolates.

The association between HIV infection and the quantity of antibodies to VSAs expressed by both the placental and the pediatric isolates was also determined (Fig. 1). Sera from HIV^+ women had lower levels of antibodies to VSAs from pediatric isolates (median, 36.03 units; interquartile range [IQR], 17.07 to 52.83 units) than sera from HIV^- women (median, 66.59 units; IQR, 38.99 to 80.31 units) ($P = 0.035$). Recognition of IRBCs from placenta was also lower when sera from HIV^+ women (median, 38.95 units; IQR, 31.57 to 48.09 units) than from HIV^- women (median, 58.55 units; IQR, 43.62 to 73.02 units) were used, and this difference was also statistically significant ($P = 0.005$).

DISCUSSION

We previously reported that HIV^+ pregnant women have fewer antibodies to VSAs from the parasite line CS2, which expresses the *var2csa* gene, associated with placental malaria (11). In the present study of clinical isolates, sera from HIV^+ women had fewer antibodies to VSAs expressed by IRBCs from both placental and pediatric isolates than HIV^- women. More than 90% of the maternal serum samples collected near the time of delivery had detectable levels of antibodies to placental VSAs, regardless of their parity and HIV infection status, suggesting that antibodies to placenta-associated VSAs result from a single or a very few episodes of malaria in both HIV^+ and HIV^- women. Such a restricted repertoire suggests that these antibodies against placental isolates target relatively conserved epitopes and therefore enable a sufficient antibody response against pregnancy-associated malaria. Because the present study used a range of clinical isolates rather than a single laboratory-adapted isolate, we propose that it better reflects the true extent of the effect of HIV on the levels of antibodies to VSAs.

We had access only to isolates from children with severe malaria. Isolates infecting young children and causing severe disease may express VSAs different from those of isolates causing mild disease or infecting older children (5). We do not know whether the responses to isolates typically causing mild malaria in children are less affected by HIV than those that we tested here.

In previous studies, the levels of antibodies to most merozoite and sporozoite antigens of *P. falciparum* did not differ between pregnant women with and without HIV infection, with the exception of antibodies to AMA-1 and the circumsporozoite protein (1, 11). Nonpregnant adults with AIDS but not those with asymptomatic HIV infection (adults admitted to hospital with trauma) lacked antibodies to the ring-infected erythrocyte surface antigen (16). Our data, in conjunction with the available published data, suggest that HIV-induced immunosuppression exerts a significant effect on variant-specific immunity. In children, specific deficits in variant-specific immunity have been associated with subsequent clinical disease due to that variant (6). Our study was cross-sectional and was not designed to correlate immune responses with malaria parasite infection in the participants. Therefore, further studies are required to determine whether a defect in variant-specific immunity has a critical role in the susceptibility of HIV^+ persons to malaria parasite infection and disease.

Our observation that HIV^+ multigravidae were more likely than primigravidae and secundigravidae to have impaired antibody responses to pediatric isolates was unexpected. The levels of immunosuppression (as measured by CD4 counts) did not differ significantly with gravidity. It is well recognized that the difference in parasite prevalence between HIV^+ and HIV^- women is greatest in multigravidae, but this has been attributed to the impact of HIV on the development of pregnancy-specific immunity (15). In our previous study (11), the effect of HIV infection on antibodies to pregnancy-associated VSAs was greatest in the first pregnancy, perhaps because the ability to develop new immune responses is more likely to be affected than existing immunity. Observations that HIV infection in nonpregnant adults increases parasite prevalence and clinical disease and worsens the response to antimalarial therapy (9, 13, 17) may be explained by the effects of HIV infection on the memory response to malaria; multigravidae might have greater deficits in memory response because they have usually had longer durations of HIV infection. By failing to recognize pediatric isolates, HIV^+ multigravidae may be more likely to

be challenged by these isolates than HIV^- multigravidae. These pediatric isolates do not sequester in the placenta; but they could cause other complications of malaria, such as anemia, and evidence suggests that HIV infection and malaria have synergistic effects on anemia (15). Further studies are required to determine the importance of this observation.

We did not have any data on the history of malaria exposure, which may have differed between HIV^+ and HIV^- women or between women of different gravidities. Our sample size was limited both by access to clinical isolates and by technical limitations to the number of samples that we could test against each isolate. A larger study would allow more definitive conclusions to be drawn.

These findings may have important implications for the prevention of malaria in HIV^+ individuals. A lack of immunity to variants causing severe disease in children living in the same environment may translate into the susceptibility of expectant mothers to these parasite strains. Malaria prevention in HIV^+ pregnant women (and, perhaps, other HIV^+ groups) may minimize the burden of malaria caused by an HIV-related lack of variant-specific immunity.

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